Neuroanatomy and function of brain structures involved in the regulation of prolactin secretion and milk yield

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PhD theses

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INTRODUCTION

The offsprings of mammalian species are fed by milk produced in the mammary gland of their mother. Secretion of milk is a process called lactation. It has two major phases: **1**. initiation of the milk secretion is the lactogenesis; **2.** maintaining of the secretion is the galactopoiesis, in rats it is also called midlactation. Removal of milk happens in response to suckling. This neuroendocrine reflex mechanism is called milk ejection. For the milk secretion prolactin (PRL), for the milk ejection oxytocin (OXY) is responsible.

BACKGROUND

Biochemistry of PRL

Rat PRL is a protein hormone composed of a polypeptide chain of 197 amino acids. Its molecular weight (MW) is 23 kD. PRL was first discovered in the pituitary gland. It is encoded by chromosoma 17. In human serum macroprolactin (big-big PRL) with 100kD MW and big PRL with 40-60 kD MW were also identified. Their clinical significance is not clarified but they may be resposible for hyperprolactinemia.

Structures producing PRL

PRL is secreted in various tissues. In the anterior pituitary acidophilic cells, known as mammotropes or mammosomatotropes, secrete PRL. During lactation the mammotropic cells proliferate. In the last thirty years PRL and PRL-like immunoreactivities or PRL mRNA was demonstrated over ten tissues other than the pituitary gland including placenta, uterus, immune system, mammary gland, corpus luteum, prostata, testes, urethral, lacrimal, sweat glands, pancreatic islets, and finally the brain.

PRL receptor (PRL-R)

PRL-R belong to cytokine receptor family. PRL-Rs are widely distributed in rat tissues. Its mapping shows that the PRL-R isoforms are present in 17 tissues. Mammary gland is the primary target tissue for PRL action. The highest level of receptors was found on somatotropes, and decreasing number on lactotropes, then thyrotropes, corticotropes and gonadotropes.

Regulation of PRL secretion

It was demonstrated by Everett (1954, 1956) more than fifty years ago that a pituitary autograft without hypothalamic connections can maintain the pseudopregnancy and corpora lutea. He postulated the existence of a hypothalamic factor which is released into the portal blood and inhibits the PRL secretion. Soon it was realized that this inhibiting factor is dopamine (DA). DA is one of catecholamine neurotransmitters. There are neurons which use it as neurohormone. These neurons take up tyrosine and tyrosine hydroxylase (TH) converts it into dihydroxy-phenilalanine. This is the immediate precursor of DA. This is further converted into DA by aromatic L-amino acid decarboxylase. In other neurons DA serves as precursor for synthesis of norepinephrine. DA is produced in several brain regions including the hypothalamic arcuate nucleus (ARC). These neurons are involved in the regulation of PRL secretion. Three populations are identified to the rostro-caudal direction: 1. the periventriculo-hypophysial dopaminergic (PHDA), 2. the tubero-hypophyseal dopaminergic (THDA) and 3. the tubero-infundibular dopaminergic (TIDA) systems. The cells of origin of these pathways are located in the periventricular-ARC region. PHDA neurons are located in the most rostral subdivision and terminate in the intermediate lobe. THDA neurons occupy the middle region and terminate in both intermediate and neural lobes of the pituitary gland. TIDA neurons are located in the middle and posterior subdivisions and terminate around the capillaries in the external zone of the median eminence (ME). Under non-lactating conditions, these neurons produce DA and continuously and tonically release it into the hypophysial portal circulation. DA acts on D2 receptors of lactotropes to inhibit PRL release. When DA release is inhibited, PRL is rapidly released into the general circulation. PRL can be controlled in this manner by a host of stimuli such as stress, sexual activity and stimuli to the breast.

There are ample evidence that suggest that mammary stimulation is a powerful regulator of TIDA neuronal activity and thus, of PRL secretion. Studies of electrical stimulation of the mammary nerve of lactating rats reveal that a 3 minute stimulation produces a 63% decline in pituitary stalk and ME DA levels preceding the rise in plasma PRL.

The most widely studied neuroendocrine reflex responsible for milk production is the suckling induced PRL release (SIPR). DA acts as the main inhibitory transmitter, responsible for tonically inhibiting PRL production and release in non-lactating rats. At the beginning of lactation, suckling stimuli by the pups eventually reach the hypothalamus, inhibiting the activity of TIDA neurons, thus allowing the release of PRL from the pituitary into the general circulation and in turn, PRL stimulates milk secretion.

The exact pathway from the nipples to the neurons of the medial-basal hypothalamus that conveys the suckling stimulus to the TIDA neurons is not well characterized. Suckling also stimulates OXY release from the magnocellular supraoptico-paraventriculo-hypophyseal system. Previous reports have suggested that the release of PRL and OXY during suckling are coordinated. Studies indicate that the suckling stimulus from the mechanoreceptors of the nipples is delivered to the spinal cord with a relay in the cervical spinal nucleus. After ascending from this nucleus, a projection to the mesencephalic tegmentum conveys suckling signals to the hypothalamus for milk ejection control. There appears to be at least one additional relay before hypothalamic neuroendocrine neurons are reached. The peripeduncular nucleus (PPN), nestled among the medial geniculate nucleus, the posterior intralaminar thalamic nucleus and the cerebral peduncle, has been suggested to be an important mediator of the suckling stimulus for successful lactation. Stimulation of this nucleus was effective in releasing PRL. More medial parts of the midbrain tegmentum also released PRL, but it is unclear whether that is due to stimulation of fibers of passage or of neurons. To resolve this, it is important to distinguish the PPN from the subparafascicular parvocellular nucleus (SPFpc) or more medial regions of the tegmentum.

An interneuronal relay from the mesencephalon to the hypothalamus is proposed, but has yet to be identified for either projection to OXY or TIDA system. Since the suckling stimulus excites OXY neurons, but inhibits TIDA neurons, the pathways must diverge somewhere and based on lesions, this divergence takes place upstream from the midbrain site. It is likely that the

signals travel together until they reach the brain stem where the neuronal pathways for milk ejection and PRL regulation diverge.

In a previous study suckling stimulus induced cFos expression in some brain stem structures suggesting the role of these structures in relaying the suckling stimulus to the hypothalamus. In a study fluorogold (FG) tracer injected in the ARC was retrogradely transported to the midbrain. The tracer appeared in some cell groups in which cFos was activated by suckling stimulus. These cell groups were mainly found in the PPN and ventrolateral medulla (VLM) (Li et al 1999). In this study the tracer spread over the border of the ARC.

Several neuropeptides and neurotransmitters were identified in the cell groups which are the potential relay stations of the SIPR. In the ventrolateral part of ARC among others dynorphin (DYN) was also demonstrated in neuronal cell bodies. SPFpc was demostrated as the main source of the tuberoinfundibular peptide (TIP39) (Dobolyi et al 2003) which was isolated from the tuberoinfundibular region. In this region cell bodies were not identified, but the most dense TIP39 fiber network was shown here. Calcitonin gene related peptide (CGRP) cell bodies were also demonstrated in the SPFpc and PPN. In this region there is dense galanin (GAL) fiber network which is termination of ascending pathways from the lumbar spinal cord (unpublished data).

It was observed that soon after the initiation of suckling, DA turnover and release are markedly reduced. Overall, inhibition of the TIDA system assumes the dominant feature during suckling via marked down-regulation of the rate limiting enzyme for DA synthesis, TH (Wang et al 1993). However, the expression of TH mRNA in TIDA neurons seems to be very dynamic, reflecting the changes in suckling activity. Previous studies determined that within 1.5 hrs of termination of suckling, the TIDA neurons showed early signs of up-regulation of TH mRNA reflected by the appearance of 1 or 2 sites of heteronuclear RNA in the nucleus of TIDA neurons (Berghorn et al 2001). An increase in cytoplasmic TH mRNA was seen about 6 hours after the termination of suckling (Berghorn et al 2001) and mRNA levels peaked by 12-24 hr. Evidence of increased protein synthesis was also noted in ME terminals at 6 hr (Berghorn et al 1995). From

these data, it is uncertain if the early signs of up-regulation of TH represent a trigger for full up-regulation of TH mRNA or whether continuous stimulation of these neurons is necessary to achieve high TH levels.

Another peptide whose expression varies in TIDA neurons under non-lactating and lactating conditions is enkephaline (ENK). ENK is barely detectable in cycling rats, while its levels dramatically increase in the ARC and ME of lactating animals. The data in the literature indicate that this up-regulation of ENK is due to the hyperprolactinemia of lactation. It is not clear what role ENK in TIDA neurons plays during lactation. Although existing data show that the TH producing activity of TIDA neurons is definitely suppressed, this does not mean that these neurons are not active in synthesizing other transmitters. ENK could be co-released with DA and serve to attenuate the effect of DA on lactotropes, raising the possibility that ENK also contributes to PRL secretion.

Autonomic innervation of the mammary gland

The nipples and the mammary gland receive not only sensory, but autonomic innervation as well. Milk secretion is iniciated by PRL release and milk ejection is induced by OXY release; however, the milk yield at the beginning of the suckling is basically influenced by noradrenergic input. It was demonstrated by Findlay and Grosvenor (1969) that catecholamines depressed the milk yield at the beginning of suckling antagonizing the effect of OXY. It was also shown that β-adrenergic blocker propranolol given intracerebroventricularly (*icv)* enhanced the milk yield (Morales et al 2001). It was supposed that the β-adrenergic blocker relieved the effect of OXY on the ductal constriction in the mammary gland. Trans-section of the spinal cord between T3 and T4 segments or pharmacological sympathectomy, but not adrenalectomy and hypophysectomy, results in a faster rate of milk flow. The above-mentioned results suggest that the inhibition of milk yield at the beginning of suckling is mediated by a reflex pathway closed in the central nervous system. The afferent limb may be the same as the SIPR pathway, the efferent limb may be provided by the sympathetic pre- and postganglionic neurons present in the intermedio-lateral cell-column in the

spinal cord and in the sympathetic trunk, respectively. The central synaptic station or stations may be in the brain stem and in the hypothalamus, probably in PV.

It is well known that the postganglionic parasympathetic nervous system consists of cholinergic neurons. Cholinergic neurons also comprise a small population of sympathetic postganglionic neurons that innervate the sweat glands (Landis and Fredieu 1986; Schäfer et al 1997). Description of the autonomic innervation of the rat mammary gland was published by Gerendai and her coworkers (2001). It is not clarified at this moment what kind of neurotransmitters mediates the sympathetic stimulus to the alveoli and ductal wall of the mammary gland, which is cholinergic and which is adrenergic.

AIM OF EXPERIMENTS

Morphological studies on SIPR pathway

The aim of these morphological studies was to further clarify which of the mesencephalic nuclei provides a relay to the ARC or to a cell group in the vicinity of ARC. We conducted a series of tract tracing studies in non-lactating rats to determine if any direct PPN to ARC connections existed and if not, where the relay from the mesencephalon to the ARC neurons was located. Neurochemical nature of those arcuate neurons which receive ascending fibers from the mesencephalon and of those which send fibers from the mesencephalon to ARC was also investigated.

Physiological studies

The aim of the physiological studies was to compare the dynamics of changes in the expression of TH and ENK mRNA in TIDA neurons following a brief interruption of suckling (3-4 hours). We wished to elucidate 1) whether such brief interruption triggers full TH up-regulation that continues for a time after pup return then it declines (Hypothesis I) or 2) whether reinitiation of suckling immediately stops this process of up-regulation as a switch (Hypothesis II). We also planned to investigate 3) whether the time course of changes of ENK expression shows an opposite pattern to that of TH mRNA expression in the same animals and

4) whether the changes in the ENK expression well correlate with the ENK peptide synthesis.

The progression of TH expression was followed up to 24 hours after pups were returned to their dams. The dynamic changes in TH mRNA of TIDA neurons were compared with those of dams whose pups were permanently removed. In the same experimental animals we also investigated the ENK mRNA levels and followed the changes in the ENK peptide in the ME. Two more additional groups were included in this latter experiment. The animals of these groups were sacrificed 48 and 72 hours after the removal of pups.

Studies on autonomic innervation of the mammary gland

The aim of this part of the work was to further explore the multisynaptic autonomic neuronal chain that innervates the nipples and the mammary glands of lactating rats using retrograde virus labeling and to chemically characterize the neurons of the neuronal chain which may participate in the regulation of the milk yield at the beginning of suckling.

MATERIALS AND METHODS

Sprague-Dawley female rats were used for the experiments. For studying the SIPR pathway cyclic females were used, for the other two experiments primipara midlactating dams were used.

Non-transynaptic tract tracing experiments

Experiment 1 (7 animals): To determine the projections of neurons of the PPN, the primarily anterograde tracer biotinylated dextrane-amine (BDA) was administered into this area using iontophoresis and labeled fibers were looked for in the medial basal hypothalamus. Ten to 14 days following surgery the animals were anesthetized and perfused by 4% paraformaldehyde containing 2,5% acrolein. Then the brains were treated with cryoprotectant. Twentyfive μm thick frozen sections were incubated in biotin antibody. To demonstrate the final reaction product ABC Elit Kit and nickel intensified diaminobenzidine tetrahydrochloride (DAB) was used.

Experiment 2 (5 animals): The primarily retrograde tracer fluorogold (FG) was administered iontophoretically into the region of the ARC and labeled cell bodies were looked for in the peripeduncular region of the midbrain. FG was demonstrated using FG antibody and ABC Elit Kit.

To demonstrate the relation of BDA labeled fibers ascending from the injection site to TIDA and DYN neurons residing in the ARC, we conducted BDA and TH, and BDA and DYN double labeling using ABC Elit Kit**.** To demonstrate the relation of FG and CGRP or FG and GAL double labeling was carried out using ABC Elit Kit. To demonstrate the relation of FG and TIP39 immunoreactivities double labeling was carried out using indirect immunofluorescence staining.

TH and ENK expression in TIDA neurons

For this experiment adult timed-pregnant rats were used. The number of litters after delivery was reduced to eight. The dams were divided into 3 main groups with 5-6 rats investigated for each time point.

Group I: Dams continued to suckle throughout the experiment.

Group II. Pups were removed on post partum day 10 and the dams were perfused at 3-4, 6, 7-8, 10-12, 16-20, or 24-28 hours after removal.

Group III. Pups were removed on post partum day 10 for 4 hours (the time at which previous studies indicated clear heteronuclear TH mRNA up-regulation) and then pups were returned for 3-4, 6-8, 12-16, or 20-24 hours. In the case of ENK study, based on initial patterns of change (which indicated a very slow decline in ENK expression) additional groups in which pups were removed for 48 and 72 hrs were added.

Group IV. Females with diestrous II stage of estrous cycle were also included in the experiment in which ENK mRNA were determined.

To prepare the dams for mRNA analysis, they were anesthetized and perfused with 4% paraformaldehyde solution containing 2.5% acrolein. Brains were removed and transferred to a 30% sucrose solution then they were sectioned on a freezing sliding microtome at 25 µm and collected into a cryoprotectant/anti-freeze solution and stored at –20˚C. This procedure enables collection of tissue over prolonged periods of time and then storage of the sections with full maintenance of mRNA levels for

over 12 years with no decay. *In situ* hybridization was carried out using biotinylated riboprobe. To visualize the presence of TH mRNA anti-biotin immunohistochemistry was used. Parallel sections were stained for ENK immunoreactivity.

Image analysis of the mRNA

Three sections containing representative areas of the ARC were included in the analysis. The sections were placed under a Nikon Eclipse 800 microscope linked to a Cooke camera and two levels of the ARC were examined, IP Spectrum Software (Vienna, VA) installed on a Macintosh G4 computer was used for capturing and analyzing the images. The optical density (OD) for each cell containing TH or ENK mRNA determined separately.

Image analysis of the ENK peptide immunoreactivity in the ME

Three sections of the ME were analyzed per animal. The sections were placed under a Nikon Eclipse 800 microscope linked to a Cooke camera. IP Spectrum Software (Vienna, VA) installed on a Macintosh G4 computer was used for capturing and analyzing the images.

Data analysis

Results of the TH and ENK mRNA *in situ* hybridization as well as the ENK immunohistochemistry were analyzed using One-way-Anova, complemented with the Tukey-Kramer post hoc comparison analysis.

Transynaptic tract tracing experiments

Four Wistar (W) and nine Sprague Dawley (S-D) female primiparous (2-3 month old) rats between 7 and 15 postpartum days were used for the experiments. The number of litters after delivery was reduced to eight.

The dams were inoculated with a virus labeled with green fluorescence protein (GFP) in 2μl physiological saline ($8x10^8$ plaque forming unit). The virus was injected in the first and second nipple areas at right side. The animals were sacrificed at different times (2-4 days) after the inoculation.

Preparation of virus

A genetically modified pseudorabies (PRV) strain termed memGreen-PRV was used for the tracing experiments. The consruction of memGreen-PRV was described

elsewhere (Boldogkői et al 2000). Briefly, the wild type strain Kaplan of PRV was modified by elimination the gE and gI genes of the virus, which resulted in a viral tracer spreading in an esclusively retrograde direction. The gE and gI genes were replaced by a gene expression cassette encoding a membrane-bound GFP, which makes easy the identification of the virally-infected cells.

Tissue preparation

All animals were again anesthetized at the end of the experimental period and the blood was flushed out through the ascending aorta, then the animals were perfused by 4% paraformaldehyde in potassium phosphate buffer (KPB) (0.1M, pH 7.4). After 24 hour postfixation the right 2nd nipple area with the underlying mammary tissue, the right sympathetic trunk, the upper thoracic segments of the spinal cord and the brain was blocked and placed in ascending sucrose solution (10-20-30%), then embedded in Cryomatrix. Twenty μm thick sections were cut on cryostate. In all parts of the nervous system GFP labeling was looked for to demonstrate the presence of retrogradely transported virus. Sympathetic trunk, the spinal cord, medulla oblongata and the hypothalamus were immunostained for both dopamine-β-hydroxylase (DBH) and vesicular acetylcholin transporter (VAChT). The hypothalamus was also stained for OXY. The mammary tissues were stained for S-100 protein, CGRP, DBH and VAChT immunoreactivities.

Immunohistochemistry

After washing, the slides were treated with 1% triton X-100 for better penetration of antibodies. The following primary antisera were used for immunostaining against: *1)* VAChT (present in cholinergic nerve fibers) raised in guinea pig in a 1:500 dilution. *2)* DBH (an enzyme present in adrenergic and noradrenergic nerve fibers) raised in mouse in a 1:1000 dilution.3*)* CGRP raised in rabbit in 1:2000 dilution. Antigen-antibody complex was visualized using indirect immunofluorescence technique or ABC Elite Kit and nickel intensified DAB.

The specificity test: Omitting the primary antibodies prevented the immunostaining. We also used positive controls such as the brain stem in the case of DBH and S-100 and anterior horn of the spinal cord in the case of VAChT, where the positive staining was well established.

RESULTS

I. Morphological studies of the SIPR pathway

*Experiment 1**:** Anterograde tracer (BDA) injection in the rostral mesencephalon*

1/1. Connection between the mesencephalon and ARC. BDA administered exclusively into the PPN failed to label any fibers inside the ARC; however, labeled fibers were found in the vicinity of the ipsilateral ARC and the ventromedial hypothalamic (VMN) nuclei. Administration of the tracer ventral and medial to the PPN actually did label ascending fibers in the ipsilateral ARC. This mesencephalic area was identified as SPFpc.

1/2. Interaction between BDA fibers of mesencephalic origin and DYN neurons. The hypothalamic slides containing BDA fibers were stained for DYN immunoreactivity. BDA fibers were close apposition on DYN immunoreactive cell bodies which are located in the ventrolateral part of ARC. In turn, DYN fibers innervate TIDA neurons which are located in the dorsomedial part of ARC**.**

Experiment 2. Retrograde tracer (FG) injection in the lateral ARC

In the midbrain of those animals where FG administration included the antero-ventro-lateral portion of the ARC there was FG labeling in SPFpc.

2/1. FG and CGRP double labeling suggests that the subpopulation of the cells that projects to the ARC from the SPFpc also contain CGRP.

2/2. FG injection into the ARC, which included its ventrolateral region, also labeled cells in the SPFpc that were partially TIP39 immunopositive.

2/3. FG injection into the ARC did not label cells in the SPFpc which contained GAL.

II. Physiological studies

Quantitative analysis of TH mRNA in ARC

TH mRNA levels were significantly higher by 6 hours in the group that did not get pups back than in continuously suckling controls. TH mRNA levels continued to rise after the complete termination of suckling and remained high even 28 hrs after pup removal. On the other hand, if the pups were returned after a 4 hour separation, the mean values of TH mRNA levels remained higher than in continuously suckling controls even 20-24 hrs after the resumption of suckling. By 16-20 hours after initial separation the dams whose pups were returned had significantly lower TH mRNA levels than those whose pups were removed but not returned. This indicates that returning the pups and thus the re-initiation of suckling started to suppress the TH mRNA production in TIDA neurons, although the already up-regulated levels did not return to continuously suckling levels.

Quantitative analysis of ENK mRNA in ARC

The analysis of ENK mRNA expression by measuring OD showed that expression of mRNA rose quickly after the termination of the suckling stimulus and was significantly higher than the OD in continuously suckling dams by 6 hours after pup-removal. After reaching peak levels around 7-8 hours, the levels declined and approached the continuously suckling levels. Return of the pups and thus resumption of the suckling stimulus after a 4 hour pup separation still resulted in an increasing trend in the means of expressed mRNA, although the levels were not significantly higher than those in continuously suckling dams. Unexpectedly, the OD in continuously suckling dams was not significantly higher than in cycling female rats during the diestrous II stage.

The histology of TH and ENK mRNA expressions well supports the qantitative data. In the dorsomedial part of ARC of continuously lactating dams only a few TH expressing cells were observed. The density of the reaction product was very low. Twenty four hours after removal of the pups the TH mRNA expression was extremly enhanced. The ENK mRNA expression was very low in diestrous rats, but extremly enhanced in continuously lactating rats and it was even stronger in the ARC of rats 8 hours after removal of pups.

Quantitative analysis and histological appearance of ENK peptide in the ME

The ENK immunostaining in the ME revealed a low ENK peptide level in cycling diestrous rats and a dramatically elevated level in continuously lactating dams as it is indicated by the difference in OD and by the histological appearance. The elevation of OD is statistically significant compared to diestrous rats. 3-4 hours after the removal of pups, the peptide content of the ME dropped and continued to drop nearing diestrous levels by the end of the experimental period. The decrease became significant about 16 hours later .

When we examined the magnitude of changes in the ENK protein in the ME it was found that in continuously suckling dams the magnitude was about 9 fold higher than in cycling diestrous rats and after pup removal it declined gradually.

III. Autonomic innervation of the mammary gland

Virus labeling at the various levels of the nervous system

When the animals were sacrificed two days after the injection, GFP labeling was observed in the ipsilateral upper thoracic paravertebral sympathetic ganglia (PvG). Ventral rootlets at the corresponding levels were also labeled. In the spinal cord labeling was observed in the ipsilateral lateral horn. A considerable number of labeled cells were seen in Th2-Th5 segments, and just a few in Th6 segment. Below this level there was no labeling in the lateral horn. When the animals were sacrificed three days after the injection, a few labeled neuronal cell bodies appeared in the brain stem and the hypothalamus. When the animals were sacrificed four days later many labeled cells were seen at both sides in the VLM and scattered cells in other brain stem region including locus ceruleus, raphe nuclei, periaqueductal gray matter. Many labeled cells were observed in the PV at the ipsilateral side and only a few at the contralateral side.

Chemical characterization of the virus labeled neuronal perikarya in PvG

In the PvG there were many small size DBH immunoreactive and a few large VAChT immunoreactive neurons and a dense network of VAChT immunoreactive fibers. A subpopulation of GFP conjugated virus labeled perikarya showed DBH immunoreactivity. The DBH immunoreactive material filled out the cells.

Chemical characterization of the nerve fibers in the mammary gland

Nerve fibers in the wall of vessels of the nipple and mammary gland showed DBH immunoreactivity; however, DBH fibers were not present between the alveoli and in the wall of ducts. VAChT immunoreactive fibers were observed neither in the alveoli and ducts of the mammary gland nor in the wall of vessels.

DISCUSSION

I. Morphological findings for SIPR pathway

Our BDA injections confined to the PPN failed to label any fibers in the ARC, but did label cells just in the vicinity of ARC and in the ventromedial nucleus (VMN). The PPN, however, could still be a very important nucleus in the process of lactation, especially since it has been shown to be activated by the suckling stimulus as well as by exteroceptive stimuli from pups such as visual, olfactory and auditory in the absence of suckling. Unilateral chemical or radiofrequency lesioning of the PPN on post partum day 7 showed impairment to lactation that was due to deficient oxytocinergic activity (Factor et al 1993). Another study showed that hemitransection of the midbrain tegmentum, including the region of PPN, only blocks the milk ejection reflex from contralateral suckling (Wang et al 1996). The results of our tracing experiments from the PPN suggest that there is a PPN-VMN projection. The VMN has been known to play a role in the control of eating, as well as certain aspects of behavior. This could mean that the PPN is involved in conveying the suckling stimulus to the VMN, and thus promotes hyperphagia, which is a typical metabolic response during nursing.

The SPFpc consists of horizontally oriented cells and extends rostromedial to caudolateral direction and overlies the medial lemniscus. BDA injections confined to the SPFpc did label fibers in the ventrolateral part of the ARC suggesting direct connection between the two nuclei. Double labeling with TH revealed that the cells contacted by these fibers are not TIDA cells. Therefore, these neurons of the ARC are probably just a relay population to TIDA cells. Previous experiments suggest that about 70% percent of TIDA neurons are innervated by DYN containing axons

(Fitzsimmons et al 1992). We hypothesized that the BDA labeled axons in the ventrolateral part of the ARC, originating in the SPFpc, are actually terminating on DYN neurons.

The injection of the retrograde tracer FG confined to the ARC resulted in labeled cells in the SPFpc of the midbrain, ventral and medial to the PPN where Dobolyi and his coworkers (2003) described TIP39 neurons. Double immunostaining also showed that many TIP39 cells in the SPFpc are CGRP positive as well. Our retrograde FG injections did label TIP39 as well as CGRP positive neurons in the SPFpc, just over the medial lemniscus.

In summary, the previously proposed midbrain nucleus that plays a role in the regulation of PRL secretion via the ARC during lactation does not seem to be the PPN. Instead, we propose that the adjacent SPFpc may be the relay of the suckling stimulus to the ARC in lactating rats. We propose that the pathway of SIPR consists of 6 neurons: dorsal root ganglion, posterior horn of the spinal cord (Rexed lamina 4-5), lateral cervical nucleus, SPFpc, ventrolateral ARC, and finally the TIDA neurons. One more relay neuron in ARC is also supposed.

II. Physiological findings: Effect of suckling stimulus on TH and ENK

The physiological results confirm the hypothesis that the suckling stimulus is an important regulator of TH expression in TIDA neurons. In cyclic diestrous rats the TH mRNA level is high, about ten times higher than in continuously lactating rats. A previous study (Berghorn et al 1995) showed that the increase of nuclear TH mRNA was evident as early as 1.5 hours after the removal of pups and that the heteronuclear RNA levels peaked at 3 hours, and then declined as cytoplasmic mRNA increased. Thus the selection of a 4 hour-removal period prior to pup return in the present study represented a time after which the TIDA neurons clearly were in the process of up-regulating TH.

The results suggest that this up-regulation of TH mRNA can not be disrupted immediately if pups are returned and the neuronal input from the nipples to the ARC is reestablished. From our data it seems that the program of transcriptional up-regulation begins to very slightly subside in 10-12 hours after

pups are returned and it is significantly lower in 16 hours than in the group where the pups were not returned. TH mRNA levels, however, stayed high for the duration of the experiment regardless of the resumption of suckling. Although the mRNA levels, found in dams that had not received their pups back, rose to higher levels than in dams that received their pups back and was significantly higher than in continuosly suckling controls. In theory we would have expected the decline in TH mRNA in pup-returned dams to the levels of continuously suckling dams with time based on the 6 hr half-life described for TH mRNA (Maurer and Wray 1997). Our observation indicates that re-suckling alters the stability of the TH mRNA producing machinary after being awakened by pup removal.

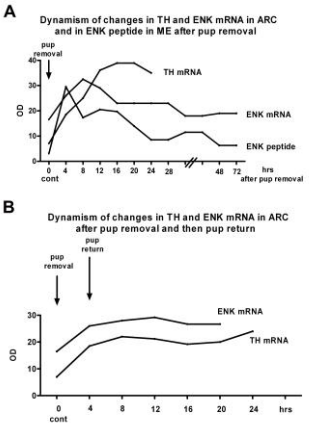
Endogenous opioids have also been implicated in the regulation of suckling-induced PRL secretion during lactation (Arbogast and Voogt 1998). A possible candidate could be ENK, a δ receptor agonist. The ARC nucleus contains scattered ENK immunoreactive neurons in cycling animals, but during lactation, ENK expression is strongly enhanced in TIDA neurons (Merchenthaler 1993). Our results examining ENK expression in the TIDA neurons also show that during continuous suckling, levels of ENK mRNA are significantly higher than in cycling females. The pup-removal produced a further increase in OD, then declined. The levels of ENK peptide in the ME started to drop earlier than the mRNA in the ARC, already four hours after the pup removal; however, the ENK mRNA only in 8 hours after pup removal. None of these parameters reached the control diestrous levels.

What kind of mechanism is responsible for maintaining ENK synthesis in TIDA neurons so long after the cessation of suckling is unclear. The up-regulation of ENK is thought to be the result of the hyperprolactinemia of lactation (Merchenthaler et al 1995). A variety of experimental paradigms shows that elevated serum PRL levels are accompanied by up-regulation of ENK. Certainly the levels of PRL fall rapidly (about 2 hours) after suckling ceases and the normal peaks in PRL secretion that accompany estrous cyclicity are not sufficient to prompt significant co-expression of ENK in TIDA neurons (Grosvenor et al 1979).

On the basis of the results it was concluded that the up-regulation of DA synthesis after termination of suckling is an active process rather than a simple

switch prompted by brief interruption in suckling. Our results support Hypothesis I. This regulatory mechanism is efficient at day 10 post partum in rats. For ENK, we can say that lactation does result in some increase in its mRNA expression; however, the translation of the message into peptide is what is truly striking. During continuous lactation ENK protein levels are high in the ME and it is released into the portal circulation in larger amounts a few hours after suckling stops. Do TIDA neurons respond to this by increasing ENK mRNA expression to allow additional ENK to be produced, or is this just a response to the stress of pup-removal? If the goal is to produce more ENK, perhaps ENK really does have a protective role against the inhibitory effect of TH (and consequently DA), whose mRNA levels also increase following the removal of the suckling stimulus. The return of pups interrupts the up-regulation of TH mRNA, but mRNA levels remain high even a day after the resumption of lactation. ENK mRNA also remains high during this time.

As it is shown in the diagram, we may say that, on one hand, after pup-removal both TH and ENK mRNA are upregulated, 8 hours later TH mRNA keeps to increase, but ENK mRNA shows opposite changes as TH mRNA, it starts to decrease (A panel). These changes show that the temporal program of TH and ENK regulation is different in the case of pup removal, and ENK response is more slow than that of TH. Upon pup return four hours later both TH and ENK mRNA remain higher than in continuously lactating rats (B panel). The curves in the figure below showing the OD of TH and ENK mRNA run parallel that is the regulation of both TH and ENK shows similar temporal program at least for 24 hours.



III. Morphological findings for the autonomic innervation of the mammary gland

Our virus labeling shows that the autonomic innervation of the rat mammary gland follows the general rules. Postganglionic neuronal cell bodies are present in the PvG. The preganglionic neuronal cell bodies are present in the corresponding part of the lateral horn of the spinal cord. The neurons in the lateral horn receive afferents from the brain stem and hypothalamic PV. These findings well correlate with those demonstrated by Gerendai and her coworkers (2001).

Chemical characterization of the virus labeled neurons in the PV revealed that the subpopulation of these neurons synthetize OXY. It is well known from the literature that OXY release is modulated by various stressors, such as immobilization and psychological stress. It is also demonstrated that acute physical and mental stress can impare the milk ijection reflex by reducing the release of OXY. It was published by Michaloudi and his coworkers (1997) that the oxytocinergic magnocellular neuronal somata were heavily innervated by noradrenergic fibers. Our virus labeling and double labeling immunohistochemistry clearly show that the first-order neurons of the descending pathway from the hypothalamus to the mammary gland is partially oxytocinergic and it is also clear from the literature that these neurons are heavily innervated by noradrenergic fibers (Semeniken et al 2009). This finding well correlates with the results that central

administration of β-adrenergic blocker propranolol enhanced the milk yield through relieving the OXY provoked ductal constriction (Morales et al 2001). It means that in this condition noradrenalin is inhibitory for the OXY release.

The further characterization of the descending neuronal chain to the mammary gland and the nipple confirmed that the second-order neurons in the lateral horn of the spinal cord are cholinergic. The third-order neurons in the PvG labeled by virus are also noradrenergic .

In the second part of the experiment we studied the innervation of the nipple and mammary gland. We did not find CGRP fibers between the alveoli or the wall of ducts, just in the connective tissue of the nipple, in the wall of vessels and under the epithelium. DBH immunoreactive fibers were only present in the wall of the vessels. It explains the colocalization between the virus labeling and DBH immunoreactive neurons in the PvG and also explains the early observation of Grosvenor and Findlay (1968) that the denervation influences the fluid flow into the mammary gland. We did not find DBH or VAChT immunoreactive fibers in the mammary gland, either between the alveoli or in the wall of the ducts.

In summary it was concluded that the descending pathway, which provide the autonomic innervation of the structures of nipple and the mammary gland and may be involved in the regulation of milk yield in rats, is composed of at least three neurons. The first-order neurons are mainly located in hypothalamic PV, and these neurons are partially oxytocinergic and they receive noradrenergic input. First-order neurons may also occur in some brain stem nuclei. These cell groups are known to be noradrenergic. The second order neurons are present in the lateral horn, and these neurons are cholinergic. The last (third-order) neurons are located in the paravertebral sympathetic trunk and these neurons are noradrenergic. Noradrenergic fibers innervate vessels and in this way may influence the blood supply of the mammary gland. Neither noradrenergic nor cholinergic fibers were seen in the wall of ducts and between the alveoli.

Our results provide morphological basis of the previous theory that the milk yield at the beginning of suckling is mainly influenced by central effect of noradrenergic input and at this level influence the OXY release from the posterior

pituitary to the general circulation. An alternative theory is also arised: descending oxytocinergic influence may modify the function of the lateral horn neurons. It was supposed that the balance of supranuclear oxytocinergic input from the PV neurons through the descending noradrenergic input, which regulates the blood supply, and hormonal oxytocinergic input from the posterior pituitary may regulate the milk yield of the rat mammary gland at the beginning of suckling.

NEW RESULTS

1) The ascending sensory pathway from the mammary gland is composed of at least six neurons. Previously it was proposed that the mesencephalic relay neurons are located in the PPN. On the basis of our results it was suggested that the mesencephalic relay is not only the PPN, but the SPFpc. **2)** It was also demonstrated at the first time that the neurons of SPFpc project to the ventrolateral part of ARC and this ascending neurons are TIP immunoreactive. **3)** In the ARC we have found DYN immunoreactive neurons which showed interaction with ascending fibers from the SPFpc. This result suggests that DYN neurons relay the suckling stimuli to TIDA neurons. **4)** Our present results show that the up-regulation of DA synthesis after suckling termination is an active process rather than a simple switch prompted by brief interruption in suckling. **5)** Lactation does result in some increase in ENK mRNA expression. During continuous lactation ENK peptide levels are high in the ME and it is released into the portal circulation in a large amount in a few hours after suckling stops. The role of this high ENK release may be protective response to pup removal. **6)** The descending autonomic pathway which innervates the blood vessels of the mammary gland and influences the milk yield at the beginning of suckling is composed of three neurons. This three parts of the pathway uses 1) OXY and noradrenaline, 2) acethylcholine and 3) noradrenaline neurotransmitters, respectively.

BRIEF CONCLUSION OF THE THREE EXPERIMENTS

I. An ascending pathway from the nipple to the hypothalamic TIDA neurons regulates the PRL secretion which induces milk secretion. In the present work the neuronal chain of this pathway was further clarified. SPFpc and the ventrolateral part of ARC were identified as relay stations.

II. A descending autonomic pathway regulates the milk yield at the beginning of suckling. This pathway is under noradrenergic influence. In this work the neurotransmitters of this pathway were further clarified. In the mammary gland in rats only the vessels are innervated by autonomic fibers which use noradrenaline as neurotransmitter.

III. TH and ENK mRNA levels in the ARC of lactating rats run parallel after removal of pups from the mother that is the regulation of both TH and ENK shows similar temporal program at least for 24 hours after removal of pups.

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